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Chromate-induced human erythrocytes haemoglobin oxidation and peroxidation: influence of vitamin E, vitamin C, salicylate, deferoxamine, and *N*-ethylmaleimide

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Abstract

In order to attenuate or to prevent chromate-induced human erythrocytes injury, the influence of vitamin E, vitamin C, salicylate, deferoxamine, and *N*-ethylmaleimide on chromate-induced human erythrocytes haemoglobin oxidation and peroxidation were investigated. It was observed that pretreatment of human erythrocytes with vitamin E (20 μM), vitamin C (1 mM), salicylate (3 mM), and deferoxamine (4 mM) significantly increased ($P = 0.0001$) chromate-induced human erythrocytes haemoglobin oxidation in a time dependent manner, while it was significantly decreased ($P = 0.0001$) by pretreatment with *N*-ethylmaleimide (1 mM). In contrast, pretreatment of human erythrocytes with deferoxamine (4 mM) immediately inhibited ($P = 0.0001$) chromate-induced human erythrocytes peroxidation, while it was significantly increased ($P = 0.0001$) by pretreatment with *N*-ethylmaleimide (1 mM) during the first 4 h of cells exposition to chromate. For time periods superior to 6 h pretreatment with *N*-ethylmaleimide (1 mM) significantly decreased ($P = 0.0001$) chromate-induced human erythrocytes peroxidation. It was concluded that care must be taken as these drugs are used to prevent against toxicity induced by chromium(VI) compounds.

Keywords: Antioxidants; Chromate; Cytotoxicity; Haemoglobin oxidation; Human erythrocytes; Oxidative stress

1. Introduction

Chromium (VI) is a widespread toxic metal ion present in industrial effluents and wastes. The toxic and carcinogenic effects of chromate have been attributed to chromium(VI) compounds, which readily cross the cell membranes and enter the cells, being reduced through reactive intermediates such as chromium(V) and chromium(IV) to the more stable chromium(III) by intracellular reductants such as ascorbate, glutathione, and

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NADPH-dependent flavoenzymes. This reduction process also causes the generation of reactive oxygen species (ROS) via Fenton or Haber–Weiss type reactions. Because ROS can produce a number of toxic reactions including DNA damage and lipid peroxidation, the toxic and carcinogenic effects of chromium(VI) may be partially associated with the production ROS (Shi et al., 1999). Therefore, therapeutic agents that enhance intra-and extracellular antioxidant levels and block chromium(VI)-mediated generation of ROS may prevent or attenuate chromium(VI)-induced toxicity and carcinogenesis (Shi et al., 1999).

The role of physiological antioxidants, namely vitamin E and vitamin C, as well as the role of the membrane-permeable metal-chelating agent deferoxamine in chromium(VI)-induced cellular injury, including cytotoxicity, and lipid peroxidation has been studied in mammalian cells and experimental animals (Sugiyama, 1992; Susa et al., 1996, 1997). It has been shown that pretreatment of cultured Chinese hamster V-79 cells with vitamin E protected cells from chromate-induced cytotoxicity (Sugiyama et al., 1989). By contrast, treatment of these cells with vitamin C enhanced chromate-induced cytotoxicity (Sugiyama et al., 1991). Although pretreatment of isolated rat hepatocytes with vitamin E or vitamin C protected the cells from chromium(VI)-induced lipid peroxidation (Ueno et al., 1989), vitamin E had no effect on chromate-induced cytotoxicity (Ueno et al., 1989). Moreover, pretreatment of primary cultures of rat hepatocytes with vitamin E (Susa et al., 1996) or deferoxamine (Susa et al., 1997) protected cells from both chromate-induced cytotoxicity and lipid peroxidation. In animals studies, it has been shown that dietary pretreatment of rats or guinea pigs with vitamin E (Chorvatovicová et al., 1991) or vitamin C (Ginter et al., 1989; Chorvatovicová et al., 1991) protected bone marrow cells from chromium(VI)-induced cytoxicity. However, both vitamin E and vitamin C dietary intake had no effect on liver peroxidation of chromium(VI)-intoxicated rats (Ginter et al., 1989; Chorvatovicová et al., 1991). Other studies, reported that vitamin C protected the liver and the kidney of mice treated with chromium(VI) from chromate-induced peroxidation (Susa et al., 1989). It has also been shown that the toxic effect of chromium(VI) on liver microsomes was pronounced in guinea pigs with marginal vitamin C deficiency (Ginter et al., 1989).

Previous studies performed in our laboratory showed that chromate-induced haemoglobin oxidation and membrane peroxidation in human erythrocytes (Alpoim et al., 1995; Fernandes et al., 1999). It also promoted oxidation of GSH, inhibition of glutathione reductase and methaemoglobin reductase, and the echinocytic shape transformation of erythrocytes. However, chromate was without effect on the activities of catalase, gluthatione peroxidase, and superoxide dismutase, as well as on the osmotic fragility of the cells (Fernandes et al., 1999). Based on these findings it was suggested that chromate may be cytotoxic to human erythrocytes. It should be taken into account that in human erythrocytes the intracellular redox balance glutathione reduced/glutathione oxidized, haemoglobin/methaemoglobin ratio, and the cell shape, which are crucial for cell functions and survival, are irreversibly disrupted (Fernandes et al., 1999).

In order to attenuate or to prevent chromate-induced human erythrocytes injury, in the present study we tested the influence of antioxidants, namely vitamin E, vitamin C, and salicylate, a phenolic compound with ability to scavenge hydroxyl radicals (Gassen and Youdim, 1999), as well as the influence of the membrane-permeable metal-chelating agent deferoxamine (Romero et al., 1996), and that of the thiol groups blocker *N*-ethylmaleimide (Snyder et al., 1988; Sullivan et al., 1992) on the chromate-induced human erythrocytes haemoglobin oxidation and peroxidation. *N*-ethylmaleimide was used because blocking thiol groups may decrease chromium(VI)-reduction by intracellular reductants and, consequently, the cytotoxic effects of chromate.

2. Materials and methods

2.1. Chemicals

All chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA) except

sodium dichromate and deferoxamine, which were purchased from Aldrich (Milwaukee, WI, USA) and Ciba–Geigy (Basel, Switzerland), respectively.

2.2. Preparation of human erythrocyte suspensions

Venous blood (20 ml) was obtained from healthy volunteers into blood collection tubes containing acid citrate dextrose as anticoagulant. The blood was centrifuged at $600 \times g$ in a bench centrifuge (Sigma-302) for 5 min at 4°C. The plasma and buffy coat were removed and the erythrocytes were washed three times with 20 ml of 0.9% NaCl solution. The erythrocytes were subsquently diluted to 30 ml with Hepes buffer (10 mM Hepes, 10 mM glucose and 140 mM NaCl, pH 7.4), and enumerated with a Cell–Dyn 1600. The number of erythrocytes in the suspension was adjusted to a concentration of approximately 3 000 000 erythrocytes/μl with Hepes buffer and the haemoglobin content measured by using the Drabkin's method (Drabkin and Austin, 1932).

2.3. Measurement of human erythrocytes haemoglobin oxidation

The human erythrocytes haemoglobin oxidation was studied in cells either untreated and pretreated with α-tocopherol succinate (vitamin E), vitamin C, sodium salicylate (salicylate), or deferoxamine (DFO) as follows: aliquots of human erythrocyte suspensions containing 2.5 mg/dm^3 of haemoglobin in Hepes buffer were incubated with vitamin E succinate (20 μM), vitamin C (1 mM), sodium salicylate (3 mM) or DFO (4 mM) at 30°C for 2 h in a final volume of 2 ml. At the end of the incubation time 4 mM dichromate were added, and the levels of haemoglobin oxidation were measured according to the method of Martinek (1965), at several time periods along 1 h of exposition of the cells to dichromate.

The human erythrocytes haemoglobin oxidation was also studied in *N*-ethylmaleimide (NEM) pretreated human erythrocytes and untreated cells. In this case, aliquots of human erythrocyte suspensions containing 2.5 mg/dm^3 of haemoglobin in Hepes buffer were incubated with 1 mM NEM in 0.9% NaCl at 25°C for 30 min, in a final volume of 20 ml. At the end of the incubation time, the cells were washed five times with 0.9% NaCl, adjusted to a concentration of approximately 3 000 000 erythrocytes/μl by dilution with Hepes buffer, and the haemoglobin content measured by the method reported above.

Controls with and without dichromate, sodium salicylate, DFO, vitamin E, vitamin C, were carried out simultaneously. Ethanol (1%) was used as vitamin E solvent. Thus, in the experiments with vitamin E, the controls were performed in the presence of ethanol (1%). The results were expressed as percentage (%) of haemoglobin oxidation.

2.4. Measurement of human erythrocytes peroxidation

The measurement of human erythrocytes peroxidation was performed in cells either untreated or treated with DFO and NEM as described above for the measurement of human erythrocytes haemoglobin oxidation. The assays were performed using 10 ml of human erythrocyte suspensions in Hepes buffer (3×10^6 cells/μl) incubated at 30°C. The reactions were initiated by the addition of 8 mM dichromate and the peroxidation levels were measured at several time periods along 8 h of exposition of the cells to dichromate. The levels of human erythrocytes peroxidation were measured using 0.5 ml aliquots of human erythrocyte suspensions. Briefly, 0.5 ml aliquots of human erythrocyte suspensions were cooled down to 0°C and 0.5 ml of 20% tricloroacetic acid (TCA) was added. The samples were vortexed, centrifuged at $600 \times g$ in a bench centrifuge (Sigma-302) for 3 min at 4°C and the supernatant carefully removed. Two ml of 0.75% of thiobarbituric acid (TBA) in 0.1 M HCl were then added to the supernatant and the mixture heated at 90°C for 15 min, recooled in ice for 10 min, centrifuged again for 15 min at $850 \times g$ and the supernatant carefully removed. The peroxidation levels were evaluated by measuring thiobarbituric acid-reactive substances (TBARS), as described by Ernster and Nordenbrand (1967). The results were ex-

pressed as nmol of malonaldehyde per million of cells (nmol MDA/10^6 cells).

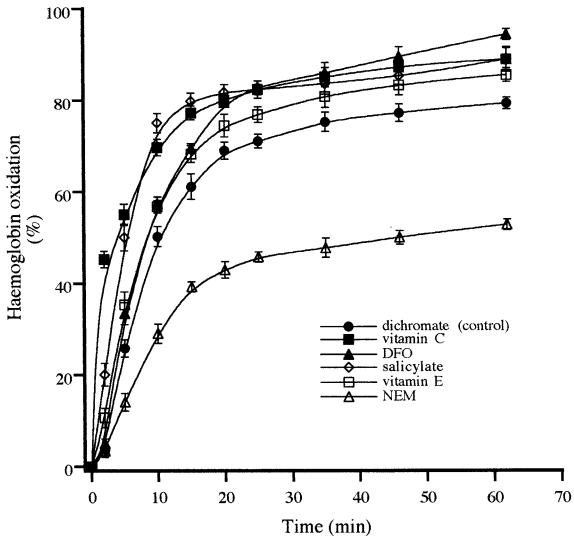

Fig. 1. Time dependent effects of vitamin E, vitamin C, salicylate, DFO and NEM on 4 mM dichromate-induced human erythrocytes haemoglobin oxidation. Results correspond to means ± SD of at least three samples carried out in duplicate.

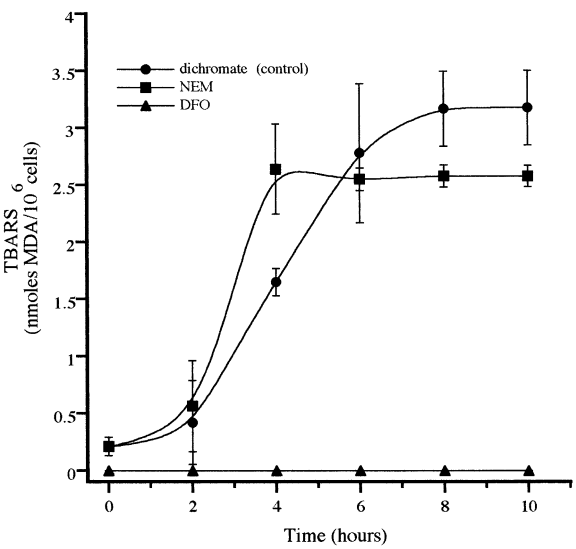

Fig. 2. Time dependent effects of DFO and NEM on 8 mM dichromate-induced human erythrocytes peroxidation. Results correspond to means ± SD of at least three samples carried out in duplicate.

2.5. Statistical analysis

All the experiments were performed using at least three different blood samples and conducted in duplicate. The values are expressed as means ± SD. Means were compared using ANOVA. Multiple comparisons with the PLSD Fisher and *F*-Scheffe-test were performed when ANOVA showed significant results. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Time dependent effects of vitamin E, vitamin C, salicylate, deferoxamine, and N-ethylmaleimide on dichromate-induced human erythrocytes haemoglobin oxidation

The time dependent effects of vitamin E (20 μM), vitamin C (1 mM), salicylate (3 mM), DFO (4 mM), and NEM (1 mM), on 4 mM dichromate-induced human erythrocytes haemoglobin oxidation are shown in Fig. 1. It was observed that pretreatment of human erythrocytes with vitamin E, vitamin C, salicylate, and DFO significantly increases ($P = 0.0001$) dichromate-induced human eryhrocytes haemoglobin oxidation in a time dependent manner, while it was significantly decreased by pretreatment with *N*-ethylmaleimide ($P = 0.0001$). Vitamin E, vitamin C, salicylate and DFO alone have no effect on human erythrocytes haemoglobin oxidation.

3.2. Time dependent effects of deferoxamine and N-ethylmaleimide on dichromate-induced human erythrocytes peroxidation

The time dependent effects of DFO (4 mM) and NEM (1 mM) on 8 mM dichromate-induced human erythrocytes peroxidation are shown in Fig. 2. It can be observed that pretreatment of human erythrocytes with DFO immediately inhibits ($P = 0.0001$) dichromate-induced human erythrocytes peroxidation. In turn, pretreatment of human erythrocytes with NEM significantly increases ($P = 0.0001$) dichromate-induced human erythrocytes peroxidation during the first 4 h of

erythrocytes exposition to dichromate, decreasing it significantly ($P = 0.0001$) for exposition times above 6 h. Deferoxamine and NEM alone have no effect on human erythrocytes peroxidation.

It is important to remark the following: firstly, under the assay conditions (pH > 7 and dichromate concentration below 10^{-1} M), dichromate depolymerise giving rise to chromate ions (Mahan and Myers, 1987), which readily cross the erythrocyte membranes and enters the cells (Connett and Wetterhahn, 1983). Thus, when we say 'exposition of the cells to 4 mM and 8 mM dichromate' it means that the cells were exposed to 8 mM and 16 mM chromate, respectively. In this study both the terms 'dichromate' and 'chromate' were used. The term 'dichromate' refers to sodium dichromate, and it appears in the Section 2, Section 3 and Figs. 1 and 2. The term 'chromate' referes to chromate ions, and it appears in the Abstract, Section 1 and Section 4; secondly, human erythrocytes haemoglobin oxidation and peroxidation were studied using 4 mM and 8 mM dichromate, respectively. Because the levels of peroxidation induced by 4 mM dichromate in human erythrocytes were very low and accurate values difficult to measure (results not shown), in the present investigation we used 8 mM dichromate instead of 4 mM to study human erythrocytes peroxidation; thirdly, the concentrations of salicylate (3 mM) and DFO (4 mM) were the lowest that, under the assay conditions, increased significantly dichromate-induced human erythrocytes haemoglobin oxidation (results not shown); finally, from the drugs tested, DFO and NEM were those with the major promoting and inhibitory effects, respectively, on dichromate-induced human erythrocytes haemoglobin oxidation. Therefore, they were selected to study their effects on dichromate-induced human erythrocytes peroxidation.

4. Discussion

The aim of this study was to investigate if pretreatment of human erythrocytes with vitamin E, vitamin C, salicylate, DFO and NEM is able to attenuate or to prevent chromate-induced human erythrocytes injury, namely cytotoxicity evaluated by haemoglobin oxidation, and peroxidation evaluated by TBARS.

The present results demonstrate that pretreatment of human erythrocytes with vitamin E (20 μM), vitamin C (1 mM), salicylate (3 mM) and DFO (4 mM) potentiate chromate-induced human erythrocytes cytotocicity in a time dependent manner, while it is attenuated by NEM (1 mM). In contrast, pretreatment of human erythrocytes with DFO (4 mM) immediately prevented chromate-induced human erythrocytes peroxidation, while it was potentiated by pretreatment with NEM (1 mM) during the first 4 h of exposition of human erythrocytes to chromate. For exposition times superior to 6 h pretreatment of the cells with NEM (1 mM) attenuated chromate-induced peroxidation.

In our recent study, it was suggested that during the intracellular human erythrocytes chromium(VI) reduction to chromium(V) intermediates, by glutathione, glutathione reductase and NADH-methaemoglobin reductase, ROS are produced via the Haber–Weiss cycle or through a Fenton-like reaction (Fernandes, et al., 1999). It was also suggested that chromate-induced human erythrocytes haemoglobin oxidation is due to the electron transfers between the haemoglobin-Fe^{2+} and chromium(V) intermediates, and that chromate-induced haemoglobin oxidation protects the cells against chromate-induced peroxidation (Fernandes et al., 1999). The oxidation of haemoglobin (Alpoim et al., 1995), which is the main protein inside human erythrocytes will readily reduce chromium(VI) and chromium(V) to the stable oxidation state chromium(III), which remain bound to the oxidized protein (Connett and Wetterhahn, 1983) not allowing ROS generation.

The finding showing that pretreamentss of human erythrocytes with vitamin E, vitamin C, salicylate and DFO potentiates chromate-induced cytotoxicity, together with that showing that pretreatment of the cells with DFO prevents chromate-induced peroxidation, indicates that these drugs potentiate the electron transfers between the haemoglobin-Fe^{2+} and chromium(V) intermediates, decreasing chromium(V) intermediates -mediated generation of ROS via the Haber–Weiss cycle or through a Fenton-like reaction. In fact, it

has been reported that cellular pretreatment with vitamin E (Sugiyama et al., 1989) or vitamin C (Sugiyama, 1991) decreases the levels of chromium(V) intermediates within cells treated with chromium(VI), while DFO supresses the levels of chromium(V) intermediates formed during reduction of chromium(VI), resulting in a decrease of chromium(V) intermediates -mediated hydroxyl radical formation (Shi and Dalal, 1992; Shi et al., 1992). Although there is no information concerning the effect of salicylate on chromium(VI)-reduction salicylate, like vitamin E, vitamin C, and deferoxamine (Morehouse et al., 1987; Gassen and Youdim, 1999; May, 1999), has an hydroxyl group with abilities to chelate chromium and to participate in oxidation-reduction reactions (Carey, 1996). Therefore, it is possible that salicylate supresses the levels of chromium(V) intermediates formed during reduction of chromium(VI) by a process similar to those described for vitamin E, vitamin C and DFO.

The findings showing that pretreatment of human erythrocytes with NEM attenuates chromate-induced cytotoxicity and potentiates chromate-induced peroxidation during the first 4 h of cells exposition to chromate, indicates that NEM decreases the electron transfers between the haemoglobin-Fe^{2+} and chromium(V) intermediates increasing chromium(V) intermediates -mediated generation of ROS via the Haber–Weiss cycle or through a Fenton-like reaction in cells exposed to chromate during this time period. However, in NEM pretreated cells exposed to chromate for time periods superior to 6 h chromate-induced peroxidation was attenuated. This may be achieved by decreasing the content of chromium(V) intermediates in human erythrocytes as a consequence of lowering chromium(VI)-reduction by intracellular thiol-reductants.

The protective or stimulative mechanisms of vitamin E, vitamin C, salicylate, DFO and NEM to chromate-induced human erythrocytes cytotoxicity and peroxidation are not yet known. In spite of this, it is possible to speculate that these mechanisms rely on drug abilities to change either the reduction potential of chromium(V) intermediates, affecting the electron transfers between the haemoglobin-Fe^{2+} and chromium(V) intermediates, or the generation of chromium(V) intermediates during chromium(VI)-reduction by intracellular reductants, affecting the levels of intracellular thiol-antioxidants such as glutathione and glutathione-reductase, as well as NADH-maethaemoglobin reductase activity, which have been shown to be suppressed in human erythrocytes exposed to chromate (Fernandes et al., 1999). In fact, the results of previous studies, which reported that pretreatment of primary cultures of rat hepatocytes with vitamin E and DFO protected cells from chromate-induced cytotoxicity and peroxidation (Susa et al., 1996, 1997), showed that vitamin E normalized the levels of glutathione and vitamin C suppressed by chromate (Susa et al., 1996), and that DFO attenuated the suppression of the levels of vitamin E and C as well as the inhibition of glutathione peroxidase activity attributed to chromate (Susa et al., 1997).

In summary, pretreatment of human erythrocytes with vitamin E, vitamin C, salicylate, and DFO potentiates chromate-induced cytotoxicity, while by contrast it is attenuated by NEM. In turn, chromate-induced peroxidation is prevented by pretreatment of human erythrocytes with DFO, while it is potentiated by NEM at least in the first 4 h of exposition of human erythrocytes to chromate. Therefore, care must be taken when using these drugs as antagonist agents for chromium(VI) compounds.

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